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THE SELECTION OF ANTHRAX BACILLI FROM LIQUIDS GROSSLY CONTAMINATED WITH E. COLI

G. Gillissen & H.G. Scholz

We examined a simple method for the selection of anthrax bacilli specifically from waste waters grossly contaminated with E. coli; only a few references for such separations are available in the literature. Since in addition to anthrax bacilli a considerable number of other bacteria grew on the lysozyme and hemin containing substrate mentioned by Pearce and Powell, Morris added 4:4-diamidino-diphenoxypropane (Propamidine) to a peptone-NaCl agar. According to Light and Co. this substance is available neither in Germany nor in England. This medium inhibited the growth of the anthrax bacillus, Bacillus cereus and Proteus, as well as all other bacteria tested. The same behavior was reported for vegetative anthrax bacilli. Larger amounts of additional erythrocytes counteracted the action of the Propamidine.

The procedure described here is based on the fact that polymyxin B is added in a corresponding concentration to a synthetic medium which permits the growth of E. coli but inhibits the germination of anthrax spores. The antibiotic kills the multiplying E. coli but does not affect the anthrax spores. Polymyxin B was chosen because vegetative forms of the anthrax bacilli -- especially the spores -- are quite resistent to this substance (Boger). Anthrax bacilli can be detected very easily in a culture treated in this manner.

Materials and Methods

Test strains were the following: Anthrax bacillus strain 1-5 (44/51, 45/51, 1888, 1889, 1894), which were kindly donated by Dr. Brandis; anthrax bacillus strains 6 and 7 (MB 1. 4. 60, MB animal hygiene), which were kindly donated by Dr. Linzenheimer, and strain 8 (Hygienic Institute, Mainz),

as well as one strain each of E. coli, Bac. subtilis, B. mesentericus, Asp. flavus and Staph. albus (this last strain was not used for all tests). Suspensions of the spores were prepared by washing 8 day old agar slants with 6 ml distilled water. Vegetative bacteria were prepared from young 16-18 hr broth cultures.

According to the work of Brewer et al. on a growth medium for anthrax bacilli, a synthetic medium was prepared which permitted the growth of E. coli, but inhibited the germination of anthrax bacillus spores. This medium contained 0.1 g $CaCl_2 \cdot 6\ H_2O$; 0.01 g $FeSO_4 \cdot 7\ H_2O$; 0.05 g $MgSO_4 \cdot 7\ H_2O$; 0.03 g $MnSO_4 \cdot 4\ H_2O$; 8.0 g glucose; 5.0 g K_2HPO_4; 4.0 g KH_2PO_4 in enough water to make one liter; in addition 0.2 μg thiamine/ml; 530 μg leucine/ml and 320 μg methionine/ml were added. Lastly, polymyxin B was added to the medium in a final concentration of 100 μg/ml. For the preparation of a solid medium, agar was added in a final concentration of 2%. Other media used were nutrient agar, blood agar and a Czapek-Dox Medium. For the filtration of the bacteria suspensions a #C0 5 membrane filter which is manufactured by Membranfiltergesellschaft in Goettingen, was used.

Results

If the synthetic medium mentioned is innoculated with the experimental bacteria (final dilution of the suspension containing the spores = 10^{-3} and that containing the vegetative cells = 10^{-2}), no visible growth is obtained with the anthrax bacillus strain #8 over a period of six days, while the subcultures on agar are positive. Bac. subtilis, Bac. mesentericus, E. coli as well as Asp. flavus, however, did grow on the liquid medium. According to the data in this experiment, the germination of the anthrax bacillus spores is inhibited without killing them. If all other conditions are kept the same, but polymyxin B is added in a final concentration of 100 μg/ml, Asp. flavus and Bac. subtilis will grow. Coliform organisms, however, area already killed after 24 hours and growth of anthrax bacilli and Bac. mesentericus cannot be observed; subcultures from these plates, however, showed normal growth. Both of these species, therefore, behave very similarly in the liquid media.

The results were confirmed by a quantitative determination of the bacteria concentration in this medium containing a polymyxin B concentration of 100 μg/ml after an incubation period at 37°C. Seven agar plates each were innoculated

with 0.3 ml of an adequate earlier dilution of the culture and the number of living organisms in the cultures calculated from the number of colonies appearing on the plates, by taking into account, of course, the dilution factor.

Table I

Change in the Concentration of Living Bacteria in Synthetic Medium Containing 100 μg Polymyxin B Per ML

a) Versuchsdauer in Std.	b) Keimkonzentration pro ml × 10^3 c) E. coli	d) MB.	e) Bac. subtilis	f) Bac. mesent.
0	312	63	542	450
6	0	60,5	528	221
24	0	—	760	133
48	0	50	721	126

[Legend]: a) Incubation time; b) Bacterial concentration per ml^3; c) E. coli; d) Anthrax bacillus Strain #8; e) Bac. subtilis; f) Bac. mesent.

If mixed cultures of the specified experimental bacteria are examined in the same manner in the liquid medium with the added polymyxin, and if they are incubated for different time intervals at 37°C after having been innoculated in the form of subcultures on agar or blood agar, one arrives at a similar bacteria count relationship as was the case with the single cultures. Coliform organisms are thus eliminated even when the starting concentration is 2000 times larger than that of the anthrax bacillus spores, whereas the other spore formers indicated growth.

These results are in agreement with the determination of resistance in the dilution test. When broth was used as a medium, bacteriostasis occurred with the spores of the anthrax bacilli of strain 8 (final concentration = 10^{-3} of the spore suspension) between a concentration of 1.2 and 18.7 μg polymyxin B/ml. This agrees with the observations by Boger. The bacteriocidal values, which were determined by transfers, were in the same range. When the synthetic medium was used, the spores did not germinate and the bacteriocidal values were higher than 100 μg/ml. Bac. mesentericus spores behaved in a similar manner (in broth cultures the values were 2.9

and 10 μg/ml, respectively; in the synthetic medium no germination of spores occurred; the bacteriocidal limit was over 100 μg/ml). When Bac. subtilis spores were tested for inhibition or bacteriocidal limits in broth, high values of 47 and 93 μg polymyxin B/ml were obtained respectively. In the synthetic medium the inhibition values were in the neighborhood of 0.3 μg/ml due to a retardation in growth. The bacteriocidal limit was higher than 100 μg/ml. While with Asp. flavus limiting values of below 100 μg/ml were not observed in either of the two media, the E. coli strain was sensitive in broth cultures at 1.4 μg/ml and in the synthetic medium the bacteriostatic and bacteriocidal values were 0.2 and 1.4 μg/ml respectively. In the case of Staph. albus these limit values were 5.8 and 11.7 in the broth culture, and in the synthetic medium 1.9 and 15 μg/ml respectively.

According to these data one can state that anthrax bacilli can easily be isolated from a mixed flora with E. coli and that the presence of other bacteria species makes the separation difficult.

For this reason we decided to include the difference in the degree of resistance, which is occasionally observed when different strains are used, in order to improve the method. It was shown that on a solid medium, consisting of the synthetic nutrient solution (see above) and 2% agar, not only E. coli and Bac. subtilis spores germinated and grew in contrast to anthrax bacillus spores, but also Bac. mesentericus spores. One could expect, therefore, that Bac. mesentericus also can be inhibited on a corresponding medium as a result of its not too high resistance toward polymyxin. Since it is desirable to have enrichment data for the proof of the presence of anthrax bacilli, the following tests were performed with a membrane filter.

Twenty ml of a suspension containing 11 anthrax bacilli/ml and 209 E. coli/ml (calculated number of living organisms) were filtered through a membrane; the filtrate was then incubated for 20 hours at 37°C on the synthetic medium with 2% agar and 100 μg Polymyxin B/ml; transfers were performed to common nutrient agar and incubated again for 24 hours.

As a control for the bacteria concentration 20 ml each of anthrax bacilli and E. coli were filtered separately, retaining the same bacterial concentration as before, and the filtrate then added directly to the agar (see results in Table 2).

Table 2

Isolation of Anthrax Bacilli From an Aqueous Suspension with E. Coli with the use of Membrane Filters and a Synthetic Medium

(number of colonies per filter = per 20 ml of filtered suspension)

ⓐ Nachgewiesene Keimart	ⓑ MB ⓕ exp.	+ ⓒ E. coli ⓖ theor.	ⓓ MB (Kontrolle) exp.	theor.	ⓔ E. coli (Kontrolle) exp.	theor.
MB ⓗ	114	220	178	220	—	—
E. coli ⓘ	0	4180	—	—	ⓙ etwa 810 ⓚ (schwer auszählbar, dicht gelagert)	4180

[Legend]: a) bacteria shown to be present; b) anthrax; c) E. coli; d) anthrax bacilli (control); e) E. coli (control); f) exp; g) theoret; h) anthrax bacillus; i) E. coli; j) about 4180 810; k); (difficult to count because of close proximity).

In a further experiment, suspensions consisting of different bacteria species were prepared in distilled water. The number of living organisms was determined in an aliquot and innoculated. From every plate culture 20 ml were filtered over a membrane filter for 20 hours onto the synthetic medium containing 2% agar and 100 μg polymyxin B and then transferred to blood agar. Of the test plates, plate #1 contained per 10 ml, 10 anthrax bacillus spores, 20 Bac. subtilis spores and 20 Bac. mesentericus spores, 200 E. coli and 20 Staph. albus. Plates 2-6 served as checks on the results obtained and contained only one species of microorganism, namely 10 anthrax bacilli spores, 20 Bac. subtilis spores, and 20 Bac. mesentericus spores, 200 E. coli and 20 Staph. albus spores per ml. Plate #7 corresponded fully to plate 1; however, it contained only 1 anthrax bacillus spore per ml and plate 8 contained only 1 anthrax bacillus spore per ml, with no other bacteria (results are shown in Table 3).

A further series of tests of a similar nature served to verify previous results and to determine whether the results in regard to Bac. subtilis improved by longer incubation (3 days) of the filtrate on the polymyxin containing substrate. The data from this series of tests were again similar to those in the series of tests mentioned above.

Table 3

Isolation of Anthrax Bacilli From Bacterial Suspensions by a Filter Method with a Synthetic Medium

ⓐ Ansatz-Nr.	ⓑ MB	ⓒ Nachgewiesene Keimarten: ⓒ Bac. subt.	ⓓ Bac. mesent.	ⓕ E. coli	ⓖ Staph. alb.
1	ⓗ etwa 380 (200)	ⓘ Gesamtkol.-Zahl (400)	0 (400)	0 (4000)	0 (400)
2	203 (200)	—	—	—	—
3	—	etwa 320 (400)	—	—	—
4	—	—	0 (400)	—	—
5	—	—	—	0 (4000)	—
6	—	—	—	—	0 (400)
7	etwa 408 (20)	Gesamtkol.-Zahl (400)	0 (400)	0 (4000)	0 (400)
8	30 (20)	—	—	—	—

The numbers stand for the number of colonies per filtrate of 20 ml.

( ) = number of colonies present theoretically

[Legend]: a) Plate; b) anthrax bacilli; c) Bac. subt.; d) Bac. Mesent.; e) Bacteria shown to exist; f) E. coli; g) Staph. alb.; h) Ca; i) total cols.;

These tests show that in this procedure E. coli, Staph. albus and Bac. mesentericus can be inhibited even if present in high concentrations.

Comments and Discussion

A procedure was presented involving enrichment methods for the selection of anthrax bacilli from aqueous suspensions grossly contaminated with other bacterial species. After filtration through a membrane filter these were added to a synthetic medium to which polymyxin B had been added and were then transferred to blood agar after an incubation of 24 hours. The number of anthrax bacilli present were thus quantitatively determined. E. coli, Bac. mesentericus and Staph. albus were also fully inhibited, even when present in high concentration. This was not the case, however, with Bac. subtilis and Asp. flavus.

The presence of molds can be disregarded, because these grow only very slowly under the culture conditions described.

Colonies of Bac. subtilis differ from anthrax bacilli on the membrane filter and on the blood agar by causing hemolysis and can also be differentiated by the color of their colonies. Anthrax bacillus colonies are whitish-yellow under these circumstances and those of Bac., subtilis are in great excess over the anthrax bacilli since the substrate shows uniform hemolysis underneath the filter. Liquids contaminated mainly with E. coli or the other bacterial species tested here can be examined relatively easily.

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